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The Occurrence and Characterization of Hemoglobin from Different Strains of Genetically Diverse, Free-Living Frankia

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THE OCCURRENCE AND CHARACTERIZATION OF HEMOGLOBIN FROM DIFFERENT STRAINS OF GENETICALLY DIVERSE, FREE-LIVING *FRANKIA*

By

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An Abstract of the Thesis Presented
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Hemoglobins have been identified in root nodules of many actinorhizal plants. When cultured *in vitro*, the actinomycete *Frankia* strain CcI3 produces hemoglobin when grown with or without supplied nitrogen. The cyanobacterium, *Nostoc commune*, also produces hemoglobin *in vitro*, although only under nitrogen-fixing, microaerobic conditions, and in less than one fifth of the explored strains/species.

The objectives of this study were to determine if *Frankia* strains $EAN1_{pec}$, ArI3, EUN1f, CcI.17, and CcI3, members of diverse genogroups, are capable of producing hemoglobin *in vitro*, to characterize the oxygen kinetics of the hemoglobin, and to determine the effect of nitrogen, oxygen, and carbon dioxide on the amount of hemoglobin produced.

Frankia cells were disrupted under an atmosphere of carbon monoxide and the carbonmonoxy absorption spectrum of the crude extract was used to calculate the hemoglobin concentration in the cells. Hemoglobin was present in all five strains when

grown on nitrogen-free (-N) or nitrogen-supplied (+N) medium. Thus it is likely that hemoglobins are produced by most *Frankia* strains. In four of the five strains, the hemoglobin concentrations were similar in –N and +N culture. This does not support an association between nitrogen fixation and hemoglobin expression.

Hemoglobin in crude extracts from –N cultures of $EAN1_{pec}$ was partially purified by ion exchange chromatography and then subjected to size exclusion chromatography. The molecular mass, 13.4 ± 0.2 kDa (mean ± SE, n = 3), is consistent with that of truncated hemoglobins, such as the *Nostoc* hemoglobin. The hemoglobin in other $EAN1_{pec}$ ion exchange fractions was further purified using a CO-pressurized concentration cell with a 10 kDa exclusion membrane. The absorption spectra obtained from this sample showed carbonmonoxy and oxyhemoglobin absorption peaks typical of a hemoglobin. This same sample was also used to determine the k_{off} value for oxygen; 131.2 ± 5.8 s^{-1} (mean ± SE, n = 6) and 166 ± 8.2 s^{-1} (mean ± SE, n = 7) for the hemoglobin from –N and +N cultures, respectively.

Cultures of $EAN1_{pec}$ grown at 2% and 20% O_2 showed no effect of O_2 on hemoglobin concentration in the +N treatments (p = 0.632). The –N treatments showed less growth than the +N treatments and the hemoglobin concentration was greater at 2% than 20% O_2.

Cultures of *Frankia* strain $EAN1_{pec}$ and CcI3 grew more efficiently when supplied with 0.2% CO_2 when compared to 0.0% CO_2. This suggests that addition of CO_2 to cultures could assist in *Frankia* growth at low initial densities, such as isolation from root nodules.

The very rapid oxygen dissociation rate of $EAN1_{pec}$ hemoglobin is two to three-fold faster than that of *Frankia* strain CcI3 and *Nostoc* hemoglobin, which have been proposed to function in facilitated diffusion of oxygen. If hemoglobin is localized within the small volume of the vesicle or periphery of the cells, the concentrations in the immediate region in which it is found might approach those necessary for facilitated oxygen transport.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

OCCURRENCE AND FUNCTION OF HEMOGLOBINS IN NITROGEN-FIXING ROOT NODULES AND IN MICROORGANISMS

Introduction

Frankia, an actinomycete, forms nitrogen-fixing symbioses with diverse woody plants. In most of these plants, hemoglobin is found in the symbiotic root nodules formed. In the case of *Casuarina glauca*, the hemoglobin is produced by the host plant and is thought to function in oxygen transport through the host cytoplasm to the cells of *Frankia* embedded within the host cell. However in most actinorhizal nodules, the concentration of hemoglobin is four to ten times lower and such a function seems unlikely. Recently it was discovered that a hemoglobin is present in *Frankia* strain CcI3 when grown in culture (Tjepkema et al. 2002). Thus it is possible that the low concentrations of hemoglobin present in many actinorhizal nodules are produced by *Frankia* rather than the host plant. The goal of my thesis is to determine whether hemoglobins are typical of *Frankia* grown in culture, and to determine the conditions under which these hemoglobins are produced.

Actinorhizal Symbioses

Overview of nitrogen-fixing symbioses

While many free-living members of different prokaryotic genera are capable of fixing nitrogen, the only symbiotic organisms known to reduce atmospheric dinitrogen (N_2) to ammonium are the bacteria rhizobia, cyanobacteria, and *Frankia* (Ishizuka, 1992).

The gram-negative bacteria, rhizobia, infect leguminous plants. Cyanobacteria (blue-green algae) are predominantly free-living, but can have symbiotic relationships. These include the cyanobacterium-fungus association in lichens, and the cyanobacterium-plant associations in many liverwort species, the pteridophyte *Azolla*, and members of the gymnosperm family of cycads. A single angiosperm has been found to have a symbiotic association with cyanobacteria, the subtropical plant species *Gunnera* (Postgate, 1998).

The gram positive, filamentous bacteria, *Frankia*, are capable of forming nitrogen-fixing root nodules with perennial, dicotyledonous, woody plants of 25 different genera, representing eight families (Silvester et al. 1985; Baker, 1988). *Frankia* have only been isolated in pure culture since 1978 (Callaham et. al., 1978), although the nitrogen-fixing ability of actinorhizal plants has been known for decades (Bond et al. 1954). In the symbiosis, the plant is supplied with the fixed nitrogen, and the endosymbiont is supplied with energy and nutrients (Ishizuka, 1992).

***Frankia* diversity**

Although several hundred different *Frankia* strains have been isolated, the number of strains studied taxonomically is significantly smaller. Many approaches have been used to generate groupings, but the results of some studies are contradictory and the studies often do not use the same strains. A number of approaches, including whole-cell sugar and fatty acid contents (St-Laurent et al. 1987; Simon et al. 1989), DNA-DNA hybridization (Fernandez et al. 1989; Akimov and Dobritsa, 1992), and polymerase chain reaction/restriction fragment length polymorphisms of the *nif*D-*nif*K intergenic spacer and the 16S-23S rDNA intergenic spacer (Lumini and Bosco, 1999) support the

taxonomic grouping of the strains into "host-compatibility" groups that share the genus name of the first host from which the bacterium was isolated. However, strains isolated from the same original host do not always group together. Also, the *Elaeagnaceae* and *Alnus* host compatibility groups show taxonomic groupings within their respective members.

Fernandez et al. (1989) describe nine "genomic species" based on 43 isolates from three host-compatibility groups; *Elaeagnaceae*, *Alnus*, and *Casuarinaceae*. Further analysis by Akimov and Dobritsa (1992) describes nine "genospecies" based on 27 isolates from the *Elaeagnaceae* and *Alnus* host compatibility groups. As a result, taxonomic groups describing the same strains have different group designations. For example, genomic species 1, genospecies P1, host specificity group 1 (HSG 1) (Baker, 1987), and genogroup 1 (An et al. 1985) all describe an overlapping group of *Alnus*-infective strains. Furthermore, genomic species 4 and 5 and genospecies P8 describe an overlapping group of *Elaeagnaceae*-infective strains (Lumini and Bosco, 1999). The strains chosen for this thesis include members from all three host compatibility groups and group into separate taxonomic groups in all of the above studies.

Protection of nitrogenase from oxygen

Nitrogen-fixing organisms reduce dinitrogen using the enzyme nitrogenase, which consumes large amounts of ATP (Wittenberg and Wittenberg, 1990). Cellular respiration, via oxidative phosphorylation, requires oxygen to generate this ATP. Paradoxically, nitrogenase is destroyed by free oxygen (Silvester et al. 1990).

Legume nodules achieve protection of nitrogenase from excess oxygen by a layer of cells that lack air spaces and surround the central nodule cells that contain the nitrogen-fixing bacteroids. This, coupled with the high metabolic activity of the infected cells, results in a low concentration of oxygen in the nitrogen-fixing cells (Tjepkema, 1979; Denison and Layzell, 1991). Free-living rhizobia lack such an oxygen barrier, and subsequently must be supplied with a nitrogen source when grown *in vitro* at atmospheric pO_2. The bacteroid-containing cells of legume nodules that are within the oxygen barrier have large intercellular air spaces that allow the diffusion of oxygen. Without these air spaces, the innermost cells would have pO_2 values too low for significant oxidative phosphorylation while the outer cells would have pO_2 values too high for nitrogenase activity (Tjepkema, 1979).

Most actinorhizal nodules lack a plant-derived diffusion barrier. Instead, the nitrogenase is protected from excess oxygen by "vesicles" produced by *Frankia*. Vesicles are terminal structures long known to differentiate when the bacterium is grown in nitrogen-limited environments (Tjepkema et al. 1981) and are a defining characteristic used to identify the genus (Benson and Silvester, 1993). The vesicles contain the enzyme nitrogenase (Meesters et al. 1987) and are thus the primary site of nitrogen fixation (Tjepkema et al. 1980; Noridge and Benson, 1986; Tisa and Ensign, 1987). There are some exceptions to the above statements in that nitrogenase activity has been observed in free-living *Frankia* strain CcI3 when grown at low pO_2. At sufficiently low pO_2, no vesicles are formed (Murray et al. 1985). Also, *Frankia* in *Casuarina* and *Allocasuarina* nodules do not form vesicles, yet the nitrogenase activity of *Casuarina cunninghamiana* nodules, as observed through acetylene reduction, was greater than many other

actinorhizal nodules (Tjepkema and Asa, 1987). The lack of vesicles may be explained by the presence of a specialized hydrophobic host cell-wall layer in *Casuarina* which could function as an oxygen diffusion barrier (Berg and McDowell, 1988). Finally, while ammonia-free cultures showed acetylene reduction, ammonia-supplied cultures of CcI.17 showed no nitrogenase activity, but only decreased vesicle formation by 70% (Meesters et al. 1985). This implies vesicles can be present without nitrogenase activity.

Externally surrounding the vesicle are multiple layers of hopanoid lipids, which provide the necessary oxygen diffusion barrier (Berry et al. 1993). *Frankiae* can regulate the thickness of their vesicle walls in response to changes in pO_2; free-living *Frankia* have been shown to increase the number of layers surrounding the vesicle at higher oxygen concentrations (Parsons et al. 1987). Similarly, *Alnus* nodules maintained at higher pO_2 showed thicker vesicle walls than those grown at lower pO_2 (Silvester et al. 1988).

Hemoglobins in Diverse Organisms and Possible Functions

Characteristics of the universal globin hemoproteins

Globin hemoproteins have the ability to reversibly bind oxygen. The oxygen-binding portion of the protein is a prosthetic heme group. A confusing terminology has developed over the decades of published research in that the terms hemoglobin and myoglobin have been used to describe functionally similar proteins. Hemoglobin (Hb) has been used to describe the circulating oxygen binding protein of mammals and the non-circulating, intracellular globin hemoproteins found in bacteria, yeasts, algae, protozoa, and plants. The term myoglobin is generally used to refer to the globin

hemoproteins in muscles. It is believed that their function is the facilitation of oxygen diffusion within the muscle.

A myriad of diverse organisms, from all five kingdoms, have been found to contain hemoglobin. The reported amino acid sequences of the nearly 200 non-vertebrate and over 600 vertebrate hemoglobins and myoglobins show close similarity when aligned and suggest the majority of the vertebrate and nonvertebrate hemoglobins are derived from a common ancestral 16-17 kDa hemoprotein family that functioned in oxidation-reduction reactions (Kapp et al. 1995: Moens et al. 1996). The tertiary structure, or myoglobin fold, is a three on three helical sandwich. This structure is conserved even in distantly related globin hemoproteins (Bolognesi et al. 1997) except in the case of the truncated hemoglobins discussed below.

The diversity in functions of the different hemoglobins can be attributed to differences in amino acids within the polypeptide chain of the globin portion (Suzuki and Imai, 1998). The vast diversity in oxygen binding characteristics results from differences in heme pocket structure, which is argued to have resulted from adaptation of each organism to varying functional needs since both oxygen association and dissociation rates vary widely (Wittenberg, 1992). Portions of the hemoglobin are strictly conserved; a phenylalanine at position CD1 and a histidine at position F8. Furthermore, studies on the differences in the heme pocket of typical vertebrate globin hemoproteins have shown conserved residues distal to the heme pocket; a leucine residue (position B10) and a histidine residue (position E7) or homologous histidine side chain (Weber and Vinogradov, 2001). The E7-His is highly conserved and can form a hydrogen bond with

the bound oxygen, stabilizing the ligand/pocket bond (Phillips and Schoenborn, 1981; Arredondo-Peter et al. 1998).

It is also important to note that oxygen binding affinity (K_{O2}) is determined by the ratio of oxygen association and dissociation rates; this can result in high oxygen affinity even when the association constant is moderate if the corresponding dissociation constant is very low (Weber and Vinogradov, 2001). Oxygen affinity is reported in terms of partial pressure of oxygen at 50% oxygen saturation, P_{50}. The lower the value, the higher the oxygen affinity (Suzuki and Imai, 1998). While there are many globin hemoproteins, only those most relevant to this study will be discussed in further detail below.

Nodulating plants

Within the infected cells of legume nodules there is a high concentration of a hemoglobin that has been named leghemoglobin (Lb). The function of leghemoglobin is to facilitate the diffusion of oxygen throughout each infected plant cell. This prevents excessive oxygen near the intercellular air spaces, while supplying sufficient oxygen for ATP generation to the bacteroids furthest from the air spaces (Tjepkema, 1979). This is consistent with the need to protect nitrogenase by removing excess oxygen while at the same time presenting the oxygen to the final electron acceptor in oxidative phosphorylation (Appleby et al. 1983; Appleby, 1984; Wittenberg et al. 1986; Fleming et al. 1987).

To perform this function, leghemoglobin must meet two requirements: a sufficient concentration and a high oxygen affinity. The symbiotic globin hemoproteins isolated from nodule-forming plant species have very high oxygen affinities resulting from low

oxygen dissociation rates compared to their combination rates (Gibson et al. 1989). For a hemoglobin to facilitate significant oxygen diffusion through a cell, there must be a gradient of oxygenated hemoglobin between the oxygen source and the point of oxygen consumption. Further, the concentration must be high enough that oxygen transport via hemoglobin is substantial and more efficient than that of free oxygen diffusion (Hill, 1998). Wittenberg (1992) proposed a concentration of no less than 100 μM would be necessary to perform this function.

Actinorhizal nodules have a wide variation in hemoglobin (CO-reactive heme) content. In contrast to legume nodules, which have relatively large amounts of plant-derived hemoglobin (up to 174 nmol g^{-1} fresh mass), the greatest hemoglobin concentrations recorded in actinorhizal nodules occur in *Myrica gale* (103 nmol g^{-1} fresh mass) and *Casuarina cunninghamiana* (80 nmol g^{-1} fresh mass) (Tjepkema and Asa, 1987). The remaining known actinorhizal hemoglobins are significantly less abundant in the nodule, and it is unclear whether these are plant or endosymbiont-derived, or both. In these cases, the hemoglobin concentrations are not sufficient to function in oxygen transport if evenly distributed in the nodule (Silvester et al. 1990). Hypotheses regarding localization of hemoglobin are discussed further below.

The hemoglobin discovered in the root nodules of *Alnus glutinosa* has an unclear function (Suharjo and Tjepkema, 1995). While the concentration of hemoglobin (20 μM) was lower than the minimum concentration proposed by Wittenberg (1992), the calculation of the *Alnus* hemoglobin concentration was determined from fresh mass of the entire nodule. If the hemoglobin is confined to the oxygen-limited symbiont vesicle, which makes up a small fraction of the total nodule mass, then the concentrations of

hemoglobin in the vesicle would likely be sufficient to function in facilitated transport of oxygen. If this is the case, a function in this manner would be further supported since it functions in a system already oxygen limited (Wittenberg and Wittenberg, 1990) by diffusion barriers in the host tissue, vesicle, or both.

Flavohemoglobins and *Vitreoscilla*

Flavohemoglobins (FHb) are a family of ≈43 kDa chimeric hemoglobins containing an N-terminal globin domain and a C-terminal flavin-binding domain (Weber and Vinogradov, 2001). Flavohemoglobins have been found in bacteria and yeasts, and were first reported in *Escherichia coli* (Vasudevan et al. 1991). The flavohemoglobin in *E. coli* (HMP) is expressed in aerobic and anaerobic conditions, and may function as an oxygen sensor by sequestering oxygen and protecting flavin from oxidoreduction (Poole et al. 1996). The reported oxygen affinities of the flavohemoglobins are relatively high, although many of these are not completely explored (Weber and Vinogradov, 2001).

The obligate aerobe bacterium *Vitreoscilla* contains a hemoglobin (VHb) with more amino acid identity to the N-terminal globin domains of the flavohemoglobins than to other non-vertebrate hemoglobins (Arredondo-Peter et al. 1997). The *Vitreoscilla* hemoglobin lacks the C-terminal flavin-binding domain of the flavohemoglobin and is a dimer composed of two identical 16 kDa subunits (Webster et al. 1987). The gene for this hemoglobin has been inserted into other cells and cell lines; *Rhizobium etli* (Ramirez et al. 1999), *Esherichia coli*, hamster cells, and tobacco (Bulow et al. 1999). The expression of the protein is upregulated in hypoxic conditions both naturally (Dikshit et al. 1989) and when recombined in *E. coli* (Dikshit et al. 1988). Cell proliferation was

enhanced in each case in a manner consistent with an increase in oxygen utilization. Expression of the heterologous *Vitreoscilla* hemoglobin gene in *R. etli* resulted in numerous physiological benefits; an increase in respiration, increase in efficiency of ATP synthesis, and enhanced nitrogenase activity (Ramirez et al. 1999). Increased ATP efficiency was also observed in recombinant *E. coli* trials (Tsai et al. 1996). While the exact mechanism of enhancement is not determined, a terminal oxidase function of this hemoglobin was shown via deletions of the *E. coli* terminal cytochrome oxidases in recombinant cells; in this experiment, the gene product performed the role of a terminal oxidase (Dikshit et al. 1992).

Truncated hemoglobins

Characteristics and occurrence. To date, more than 40 globin hemoproteins with amino acid sequences 20-40 residues shorter than the typical monomeric hemoglobin have been identified in the genome of more than 30 different organisms representing eubacteria, cyanobacteria, protozoa, and plants (Wittenberg et al. 2001). The literature has termed these truncated globins (Vinogradov et al. 1993), contracted globins (Shikama et al. 1995), or minihemoglobins (Suzuki and Imai, 1998). Recent studies have characterized three subsets of truncated globin hemoproteins identified mostly by deduced amino acid sequence similarities of reported genomes: GLBN, GLBO, and GLB3 (Pesce et al. 2000; Watts et al. 2001). A different group of authors identify three similar taxonomic groups: trHbN (identical to GLBN), trHbO (containing GLBO and GLB3), and trHbP (containing separate truncated hemoglobin genes from some members

of the GLBO group and truncated hemoglobin genes from bacteria not identified in other studies) (Wittenberg et al. 2001).

The GLBN truncated hemoglobins have been identified in a diverse group of organisms from different kingdoms discussed in detail below. The GLBO truncated hemoglobins are found in a number of bacteria, including some pathogens (Pesce et al. 2000; Watts et al. 2001). None of the GLBO hemoglobins have been studied in detail and identification of these have been by sequence data only. The GLB3 truncated hemoglobins have been reported in a Bryophyte and a wide range of angiosperms, including *Arabidopsis thaliana* (Watts et al. 2001). Based on amino acid similarity, the GLB3 protein is more closely related to GLBO than to GLBN (Watts et al. 2001). All of the reported truncated hemoglobins show greater amino acid similarity to other truncated hemoglobins than to vertebrate and nonvertebrate globin hemoproteins (Watts et al. 2001; Wittenberg et al. 2001).

Unlike the three on three helical orientation of the myoglobin fold more commonly found, it has been shown that the truncated hemoglobins from *Paramecium caudatum* (Pesce et al. 2000), *Chlamydomonas eugametos* (Pesce et al. 2000), and *Mycobacterium tuberculosis* (Milani et al. 2001) possess a two-on-two α-helical structure, which is also proposed to exist in the other truncated hemoglobins (Pesce et al. 2000; Watts et al. 2001; Milani et al. 2001). Furthermore, the secondary structure elements of these truncated hemoglobins are located in a different topological arrangement than the universal globin hemoprotein. Other motifs that are highly conserved in the truncated hemoglobin subfamily that are not in the larger superfamily,

include three glycine motifs virtually unknown in other globin hemoproteins (Bashford et al. 1987; Kapp et al. 1995)

The GLBN proteins are found in the nitrogen-fixing cyanobacterium *Nostoc commune* (12.5 kDa) (Potts et al. 1992), the cyanobacterium *Synechocystis* PCC 6803 (≈14 kDa) (Kaneko and Tabata, 1997; Couture et al. 2000, Scott and Lecomte, 2000), the virulent bacterium *Mycobacterium tuberculosis* H37Rv (14.4 kDa) (Cole et al. 1998; Couture, Yeh, et al. 1999), the ciliated protozoans *Paramecium* (≈13 kDa) (Smith et al. 1962; Iwaasa et al. 1989) and *Tetrahymena* (≈14 kDa) (Iwaasa et al. 1990; Takagi et al. 1993), and the green, unicellular alga *Chlamydomonas eugametos* (the mature Hb is 16 kDa, this includes 38 amino acids at the N-terminus which act as a transit peptide for import to the chloroplast) (Couture et al. 1994; Couture and Guertin, 1996). Based on similarities between *Nostoc* hemoglobin and the hemoglobin from *Frankia* strain CcI3 (14.1 kDa) (Tjepkema et al. 2002), it is likely that the *Frankia* hemoglobin also belongs to the GLBN group.

All of the sequenced GLBN truncated globins exhibit higher amino acid similarity with each other (20-45%) than with sperm whale myoglobin (Couture et al. 2000). These GLBN proteins have less than 15% identity to the other vertebrate and nonvertebrate hemoglobins (Couture et al. 1994; Moens et al. 1996). Another study places the truncated globins from *Tetrahymena*, *Nostoc*, and *Chlamydomonas* in a related group distant from the other non-vertebrate globin hemoproteins (Hardison, 1998). Furthermore, a horizontal gene transfer from a common ancestor of protozoa and algae may have resulted in the genetically distinct truncated hemoglobins of the nitrogen-fixing cyanobacterium *Nostoc commune*, protozoans *Paramecium* and *Tetrahymena*, and alga

Chlamydomonas, described in greater detail below (Moens et al. 1996; Suzuki and Imai, 1998).

The globin genome of *N. commune*, *Synechocystis*, *M. tuberculosis*, and the *Tetrahymena* species lack introns, while *C. eugametos* and the *Paramecium* species have one and three introns, respectively, of different amino acid length (Suzuki and Imai, 1998; Korenaga et al. 2000). It is unclear if these introns were gained independently or whether the ancestral gene contained up to four introns, some or all of which were lost during evolution. Considering the above and the fact that prokaryotes possess both the universal and truncated globins in different genera, it has been proposed that the truncated hemoglobins and the universal globin are separate gene families with distinct evolutionary origins (Moens et al. 1996; Suzuki and Imai, 1998; Pesce et al. 2000).

With the exceptions described below, all of the sequenced truncated globins from the GLBN group have a glutamine in the distal E7 position rather than the usual histidine found in the universal hemoglobin. They also contain a tyrosine in the B10 position rather than the usual leucine (Couture et al. 2000; Das et al. 2000; Wittenberg et al. 2001). These residues at E7 and B10 result in a hydrogen bond from each residue stabilizing the bound oxygen molecule and thus higher oxygen binding affinities than the typical vertebrate myoglobin (Couture, Das, et al. 1999; Weber and Vinogradov, 2001). Furthermore, the E7-Gln and B10-Tyr are in hydrogen bonding contact, providing extra stability to the hydrogen bond network (Das et al. 2001). An exception to the E7-Gln occurs in the GLBN of *Mycobacterium* and to the B10-Tyr in the GLBN of *N. commune* (Couture et al. 2000; Das et al. 2000; Wittenberg et al. 2001). The near ubiquitous presence of the E7-Gln in the above-listed GLBN proteins is of particular interest since

the E7 site is not occupied by glutamine in GLBO or GLB3, nor is the GLBO conserved at this site (Pesce et al. 2000; Watts et al. 2001; Wittenberg et al. 2001). Other factors contributing to oxygen affinity must be involved as well, however, since *Chlamydomonas* and *Paramecium* hemoglobins have the same residues at B10 and E7 as most of the other GLBN proteins, yet the oxygen affinity of the hemoglobin from *C. eugametos* (Couture, Das, et al. 1999; Wittenberg et al. 2001) is approximately 100 times greater than that of *Paramecium* (Smith et al. 1962; Das et al. 2000).

Frankia. A single study exists exploring the occurrence of hemoglobin in *Frankia*. It showed that the free-living cultures of *Frankia* strain CcI3 express a true hemoglobin in both nitrogen-fixing and nitrogen-supplied cultures (Tjepkema et al. 2002). The nitrogen-fixing cultures produced a 3-fold higher concentration of hemoglobin than cultures grown with supplied ammonium. The kinetic constants for the "on" and "off" rates support a function in the facilitation of oxygen over short distances, such as within the vesicle, and are in agreement with the rates reported for a truncated hemoglobin from *N. commune* (discussed in the next paragraph).

Nostoc commune. As in the vesicle of nitrogen-fixing *Frankia*, the nitrogenase of the photosynthetic, nitrogen-fixing, aerobic cyanobacterium *Nostoc commune* is also protected from oxygenic inactivation by differentiation of specialized cells, the heterocyst, which generates a glycolipid layer to provide an oxygen diffusion barrier (Wolk et al. 1994). A study using 41 strains of cyanobacteria identified either the cyanobacterial hemoglobin gene or the gene product in eight strains, with *N. commune* UTEX 574 the only member tested for both (Hill et al. 1996). The truncated hemoglobin

from *N. commune* UTEX 574 is well studied and this hemoglobin is discussed further below.

The hemoglobin (GlbN) from *N. commune* UTEX 574 (hereafter referred to by species name only) was found in both vegetative cells and heterocysts, but was concentrated in the periphery of the cells (Hill et al. 1996). This is consistent with a role in oxygen scavenging, as the diffusing oxygen would encounter the hemoglobin more easily near the cell surface. This hemoglobin might function by collecting oxygen within the heterocyst and presenting it the terminal oxidase (or might act as a portion of a terminal cytochrome oxidase complex) thus reducing diatomic oxygen to water (Hill et al. 1996; Thorsteinsson et al. 1999). In this manner, respiration could proceed even at the low oxygen tensions required for nitrogenase activity.

The truncated hemoglobin from *Nostoc* has a very high oxygen association rate (Thorsteinsson et al. 1999) with a moderate oxygen affinity that is lower than that of the reported plant hemoglobins and leghemoglobins, but higher than the oxygen affinity of myoglobin (Thorsteinsson et al. 1996). This may be due to presence of a B10-His residue (Couture, Yeh, et al. 1999) rather than the B10-Tyr found in the more than 40 other truncated hemoglobins (Couture, Yeh, et al. 1999; Wittenberg et al. 2001), since a tyrosine residue at B10 is consistent with a tighter oxygen-binding heme pocket because of its ability to form a strong hydrogen bond with the E7-Gln (Das et al. 2001). The association rate of this cyanobacterial hemoglobin is the highest reported in a study of non-vertebrate hemoglobins (Weber and Vinogradov, 2001).

It is important to note that *glbN*, the gene encoding the *Nostoc* hemoglobin, is located within the *nif* operon, which contains the genes responsible for nitrogen fixation

(Potts et al. 1992). Furthermore, a specific transcription factor (NtcA) essential for heterocyst formation binds immediately upstream of *glbN* in *N. commune* UTEX 584 (Hill et al. 1996). The fact that this hemoglobin is present only during nitrogen-limited conditions and the discovery that the highest level of expression occurred when nitrogen fixation was the greatest (Hill et al. 1996), both suggest that the role of this hemoglobin is linked to nitrogen fixation.

***Paramecium* and *Tetrahymena* species.** A number of *Paramecium* species have been shown to express a truncated hemoglobin. Early studies of *Paramecium* hemoglobin suggested that the concentrations of cytoplasmic hemoprotein were too low to effectively facilitate oxygen diffusion (Keilin and Ryley, 1953; Steers and Davis, 1979; Wittenberg, 1992). However, a recent study exploring the oxygen combination and dissociation rates found their values to be sufficient to facilitate the diffusion of oxygen at pressures found in the protozoans' naturally occurring habitats (Das et al. 2000). It was suggested that this hemoglobin provides the mitochondria with oxygen for use in cellular oxidative processes.

Tetrahymena hemoglobins isolated from two species, *T. pyriformis* and *T. thermophila*, show an oxygen affinity of 0.2 mmHg (Korenaga et al. 2000). This is a higher oxygen affinity than *Paramecium* and *N. commune* hemoglobins, but less than that of the two globins discussed below. The rate of autoxidation for *Tetrahymena* hemoglobin is nearly ten-fold slower than that of *Paramecium* hemoglobin (Korenaga et al. 2000), which may be the result of additional residues adding stability to the hemoglobin (Weber and Vinogradov, 2001). A specific function has not been proposed for *Tetrahymena* hemoglobin.

***Chlamydomonas eugametos* and *Synechocystis* PCC 6803.** The green unicellular algae *Chlamydomonas eugametos* and *C. reinhardtii* produce the only identified chloroplast hemoglobins. While the hemoglobin in *C. eugametos* is well studied, the hemoglobin in *C. reinhardtii* is only recently identified using the published genome (Wittenberg et al. 2001), and shares the highly conserved residues found in the GLBN group. The hemoglobin from *C. eugametos* is discussed further below.

The chloroplast genome contains three hemoglobin genes, two of which have been cloned and all of which are induced by light. The L1637 protein has been studied in detail and oxygen kinetics have been performed. It is this protein that is discussed further. The oxygen association rate is very rapid ($>10^7$ μM^{-1} s^{-1}) while the dissociation rate is among the lowest reported (0.014 s^{-1}). This results in an extremely high oxygen affinity (Couture, Das, et al. 1999). While the exact function of this hemoglobin is not known, the very low dissociation rate of oxygen is not consistent with a function in metabolic pathways requiring deposit of oxygen to oxygen-consuming enzymes. The concentrations of hemoglobin are not sufficient to function in significant oxygen storage, given the high concentration of oxygen generated by photosynthesis, unless the protein is confined to a very limited domain, i.e. the thylakoid membrane (Couture, Das, et al. 1999).

Like the algae above, the photosynthetic cyanobacterium *Synechocystis* PCC 6803, contains a truncated hemoglobin. The oxygen dissociation rate (k_{off} = 0.011 s^{-1}) is among the slowest reported. This value is much lower than most of the other truncated hemoglobins and nearly 8000-fold less than that of the *Nostoc* hemoglobin (Thorsteinsson et al. 1999). Although the oxygen association rate was not measured, this

low oxygen dissociation rate almost surely correlates with a very high oxygen affinity. The dissociation rates of hemoglobins from *C. eugametos* (Couture, Das, et al. 1999) and the nematode *Ascaris* (Gibson et al. 1965) are similarly low and have high oxygen affinities, 0.005 mmHg and 0.11 mmHg at 20° C, respectively. Given their different kinetic properties, it is likely that the hemoglobin in *Synechocystis* differs in function from that of the truncated hemoglobin found in nitrogen-fixing *N. commune.* It may, however, have a function similar to that of *C. eugametos,* since it has a nearly identical oxygen dissociation constant and 43% amino acid similarity (Couture, Das, et. al. 1999; Couture et al. 2000).

Mycobacterium tuberculosis. Four *Mycobacterium* species have been identified that contain truncated hemoglobins; *M. tuberculosis*, *M. smegmatis*, *M. leprae*, and *M. avium* (Couture, Yeh, et al. 1999; Pesce et al. 2000; Watts et al. 2001; Wittenberg et al. 2001). The disease-causing *Mycobacterium tuberculosis* strain H37Rv contains two hemoglobin genes, one coding for GLBN and the other for GLBO. The GLBN is kinetically characterized and discussed below. The very low oxygen dissociation rate of the GLBN protein makes its oxygen affinity among the greatest of the truncated globins (Couture, Yeh, et al. 1999). As noted earlier, this is the only member of the known GLBN proteins lacking the heme pocket-stabilizing E7-Gln, instead having a leucine incapable of forming hydrogen bonds with the ligand. Despite this, the *C. eugametos* GLBN has an extremely high oxygen affinity, emphasizing the importance of the B10-Tyr in heme pocket stabilization. Because of its high oxygen affinity and very large Hill coefficient, this cooperative, homodimeric GLBN will retain oxygen even at very low pO_2 (Couture, Yeh, et al. 1999). This may play a role in the pathology of tuberculosis, as the internal

environment of the granuloma is oxygen limited. This could provide enough oxygen for survival of the bacillus through the latency stage of the disease (Couture, Yeh, et al. 1999). The more critical function of protection against nitric oxide attack from macrophages during the latency stage is likely as well; it has been shown *in vitro* that this GLBN is up-regulated just before the stationary phase, which is believed to be similar to the latency stage *in vivo* (Couture, Yeh, et al. 1999). Also, the oxygenated form of human hemoglobin and *E. coli* flavohemoglobin convert nitric oxide into nitrate by way of the oxygenated protein (Gardner et al. 1998).

Sequence analysis of another lung-infecting pathogen, the bacterium *Legionella pneumophila*, identified a truncated hemoglobin grouping with the other GLBNs (Pesce et al. 2000; Watts et al. 2001). This hemoglobin contains the highly conserved E7-Gln unique to the GLBN group. Other work with this hemoglobin has not been reported.

In a recent study, a putative GLBN was identified in the methanotrophic (obtain all their cell carbon and energy from the oxidation of methane) bacterium *Methylococcus capsulatus* that mapped closer to *L. pneumophila* using amino acid sequence alignment than to the other GLBNs (Wittenberg et al. 2001).

Summary

Based on amino acid sequence similarity, the truncated hemoglobins are proposed to have a distinct origin from the larger globin hemoprotein superfamily. The GLBN truncated hemoglobins are a recently discovered group found in a few organisms from different kingdoms, habitats, and metabolic strategies. The reported kinetic characteristics of these truncated hemoglobins vary in oxygen rate constants and oxygen

affinity, and are thus proposed to have different specific functions that have probably adapted due to the niche of the organism. The occurrence of truncated hemoglobins in *Frankia* strain CcI3 and *Nostoc commune* is noteworthy because of the similarity between these organisms: both fix nitrogen and do so in specialized cells that protect nitrogenase from oxygen. To date, cyanobacterial hemoglobin has been identified in a limited number of explored strains. This raises the question whether hemoglobin found in *Frankia* strain CcI3 is unique or whether it is present in other strains of the genus as well. Answering this question is one of the major objectives of this thesis.

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CHAPTER II

HEMOGLOBIN IN FIVE GENETICALLY DIVERSE *FRANKIA* STRAINS

Abstract

Hemoglobin was extracted from five genetically diverse strains of *Frankia*. All five strains produced hemoglobin when grown on nitrogen-free (-N) or nitrogen-supplied (+N) medium, suggesting that hemoglobin is common in *Frankia* and is not associated with nitrogen fixation. Cultures of strain $EAN1_{pec}$ showed no effect of oxygen on hemoglobin concentration in the +N treatments when grown at 2% or 20% O_2, while -N treatments showed greater hemoglobin concentration at 2% O_2. These +N cultures produced more biomass at both oxygen levels. Cultures of $EAN1_{pec}$ and CcI3 produced more biomass when supplied with 0.2% CO_2 compared to 0.0% CO_2. The molecular mass of hemoglobin from $EAN1_{pec}$ as determined by size exclusion chromatography, 13.4 ± 0.2 kDa (mean ± SE, n = 3), is consistent with that of truncated hemoglobins. The absorption spectra obtained for the $EAN1_{pec}$ hemoglobin were typical of a hemoglobin. The oxygen dissociation rate constants for the $EAN1_{pec}$ hemoglobin from -N and +N cultures (131.2 ● 5.8 s^{-1} and 166 ● 8.2 s^{-1}, respectively) were two to three-fold faster than that of the nitrogen-fixing *Frankia* strain CcI3 and *Nostoc* hemoglobin, which have been proposed to function in facilitated diffusion of oxygen.

Introduction

Frankia, an actinomycete, forms nitrogen-fixing symbioses with diverse woody plants. In most of these plants, hemoglobin is found in the symbiotic root nodules

formed. In the case of *Casuarina glauca*, the hemoglobin is produced by the host plant and is thought to function in oxygen transport through the host cytoplasm to the cells of *Frankia* embedded within the host cell. However in most actinorhizal nodules, the concentration of hemoglobin is four to ten times lower and such a function seems unlikely. Recently it was discovered that a hemoglobin is present in *Frankia* strain CcI3 when grown in culture (Tjepkema et al. 2002). Thus it is possible that the low concentrations of hemoglobin present in many actinorhizal nodules are produced by *Frankia* rather than the host plant.

The hemoglobin from *Frankia* strain CcI3 has a molecular mass of 14.1 kDa. This is much less than the mass of about 17 kDa for the universal hemoglobin. This suggests that *Frankia* hemoglobin may belong to the growing list of truncated hemoglobins, a distinct group that has been recently characterized. To date, more than 40 truncated hemoglobins have been identified in 32 different organisms including eubacteria, cyanobacteria, protozoa, and plants (Wittenberg et al. 2001). These hemoglobins have amino acid sequences 20-40 residues shorter than the universal hemoglobin. Three groups of truncated hemoglobins have been identified based on amino acid sequence similarity: GLBN, GLBO, and GLB3 (Watts et al. 2001). A different group of authors identify three similar taxonomic groups: trHbN (identical to GLBN), trHbO (containing GLBO and GLB3), and trHbP (containing separate truncated hemoglobin genes from some members of the GLBO group and truncated hemoglobin genes from bacteria not identified in other studies) (Wittenberg et al. 2001). Based on amino acid similarity, the GLB3 protein is more closely related to GLBO than to GLBN (Watts et al. 2001). All of the reported truncated hemoglobins show greater amino acid

similarity to other truncated hemoglobins than to vertebrate and nonvertebrate universal hemoglobins (Watts et al. 2001; Wittenberg et al. 2001).

The GLBN proteins are found in the nitrogen-fixing cyanobacterium *Nostoc commune* (12.5 kDa) (Potts et al. 1992), the cyanobacterium *Synechocystis* PCC 6803 (14 kDa) (Kaneko and Tabata, 1997; Couture et al. 2000), the virulent bacterium *Mycobacterium tuberculosis* H37Rv (14.4 kDa) (Cole et al. 1998; Couture, Yeh, et al. 1999), the ciliated protozoans *Paramecium* (≈13 kDa) (Smith et al. 1962; Iwaasa et al. 1989) and *Tetrahymena* (≈14 kDa) (Iwaasa et al. 1990; Takagi et al. 1993), and the green, unicellular alga *Chlamydomonas eugametos* (the mature hemoglobin is 16 kDa, this includes 38 amino acids at the N-terminus which act as a transit peptide for import to the chloroplast) (Couture et al. 1994; Couture and Guertin, 1996). Based on similarities between *Nostoc* hemoglobin and the hemoglobin from *Frankia* strain CcI3 (14 kDa) (Tjepkema et al. 2002) it is possible that the *Frankia* hemoglobin also belongs to the GLBN group.

The truncated hemoglobins from *Paramecium caudatum* (Pesce et al. 2000), *Chlamydomonas eugametos* (Pesce et al. 2000), and *Mycobacterium tuberculosis* (Milani et al. 2001) possess a two-on-two α-helical structure, which is also proposed to exist in the other truncated hemoglobins (Pesce et al. 2000; Watts et al. 2001; Milani et al. 2001). Furthermore, the secondary structure elements of these truncated hemoglobins are located in a different topological arrangement than the universal hemoglobin. Other motifs that are highly conserved in the truncated hemoglobin subfamily that are not in the larger superfamily, include three glycine motifs virtually unknown in other globin hemoproteins (Bashford et al. 1987; Kapp et al. 1995) and a tyrosine at position B10

(Wittenberg et al. 2001). The GLBN truncated hemoglobins have a near ubiquitous presence of glutamine at position E7, which is occupied by five different amino acids in the other reported truncated hemoglobins, but never a glutamine (Pesce et al. 2000; Watts et al. 2001; Wittenberg et al. 2001).

The reported kinetic characteristics of the GLBN truncated hemoglobins vary in oxygen rate constants and oxygen affinity, and thus are proposed to have different specific functions that have probably evolved due to the niche of the organism. The occurrence of truncated hemoglobins in *Frankia* strain CcI3 and *Nostoc commune* is noteworthy because of the similarity between these organisms: both fix nitrogen and do so in specialized cells that protect nitrogenase from oxygen. To date, the cyanobacterial hemoglobin gene or protein has been found in less than one fifth of explored strains. The goal of this study is to determine whether hemoglobins occur in diverse strains of *Frankia* grown in culture, to determine the characteristics of these hemoglobins, and to determine the conditions under which these hemoglobins are produced.

Materials and Methods

Frankia cultures

The *Frankia* strains used in this study are listed in Table 1, and will be identified hereafter by strain designation. *Frankia* strains CcI3 and ArI3 were grown on modified DPM medium, originally described by Baker and O'Keefe (1984). This modified DPM contained (per liter): 0.1M potassium phosphate, 0.4 mM $MgSO_4 \cdot 7H_20$, 0.18 mM $CaCl_2 \cdot 2H_2O$, 12.5 mM propionic acid (sodium salt), 20 mM sodium succinate, 0.1 M Fe-EDTA, 1 ml microelements (containing [per liter]: 2.86g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_20$,

Table 1. Characteristics of the *Frankia* strains examined.

Strain	*Frankia* catalog No.	Host plant	GS[†]	P[‡]	Origin	Reference
$EAN1_{pec}$	ULQ130100144	*Elaeagnus angustifolia*	5	P8	Ohio, U.S.A.	Lalonde et al. 1981
ARI3	HFP013003	*Alnus rubra*	1	P1	Oregon, U.S.A.	Berry and Torrey, 1979
EUN1f	ULQ132500106	*Elaeagnus umbellata*	6	-	Illinois, U.S.A.	Lalonde et al. 1981
CcI.17	-	*Colletia cruciata*	-	P7	Netherlands	Hafeez et al. 1984
CcI3	HFP020203	*Casuarina cunninghamiana*	9	-	Florida, U.S.A.	Zhang et al. 1984

[†]Genomic species (Fernandez et al. 1989)

[‡]Genospecies (Akimov and Dobritsa, 1992)

0.22 g $ZnSO_4 \cdot 7H_20$, 0.08 g $CuSO_4 \cdot 5H_20$, 0.03 g MoO_3, 0.10 g $CoCl_2 \cdot 6H_20$, 0.05 g $NiSO_4 \cdot 6H_2O$, and 50 g KCl) and 1 ml vitamins (containing [per liter]: 10 mg thiamine HCl, 50 mg nicotinic acid, 50 mg pyroxine HCl, 10 mg folic acid, 10 mg calcium pantothonate, 10 mg riboflavin, and 240 mg biotin).

The basal growth medium used for strains $EAN1_{pec}$, EUN1f, and CcI.17 has been previously described (Tisa and Ensign, 1987a). The following was autoclaved separately and then aseptically combined with the medium prior to inoculation (per liter): 2 mM $MgSO_4$, 1mM sodium acetate, 20 mM Fe-EDTA (ferric sodium salt), and 1ml 10x MOD-MBA trace salts as previously described (Tisa and Ensign, 1987a).

Prior to autoclaving, the pH of the media was adjusted to 6.8 for $EAN1_{pec}$ and EUN1f and 6.7 for other strains. For cultures containing fixed nitrogen, a final concentration of 5 mM NH_4Cl was used. This amount of supplied ammonium is sufficient throughout the studied period as Tisa and Ensign (1987b) showed a concentration of 0.5 mM NH_4Cl was depleted after 7-8 days. Since our cultures had a ten-fold greater concentration, all cultures should contain a sufficient amount of supplied nitrogen. All cultures were inoculated from sterile –N cultures and grown at room temperature (23-27°C). *Frankia* cells were collected by passing the cultures through a 0.47μm filter (Osmonics) using vacuum filtration. To ensure that only small amounts of culture medium were retained, filtration was continued until all observable media had flowed through and the hyphal mass was dry enough to be easily removed from the filter. Freshly harvested cells were weighed immediately to minimize variation in mass caused by evaporation. To observe vesicles for each treatment, a drop of water was placed on the filter after the cells were removed. This drop was spread evenly on the filter, and the

liquid was collected, placed on a slide, and viewed at 400X. This resulted in a suspension with enough cells for vesicle observation.

Hemoglobin extraction

Frankia cells collected via filtration were placed in a 15 ml centrifuge tube (Corex No. 8441) and the headspace was immediately flushed with carbon monoxide. A 1 ml volume of CO-equilibrated KPO_4 extraction buffer (containing [per liter]: 14.09 g K_2HPO_4, 2.58 g KH_2PO_4, and 38 mg EDTA; pH 7.4) was added along with 2 g of 0.1 mm glass beads (Biospec Products, Bartlesville, OK). With larger hyphal masses (> 0.2 g), 1.5 ml of buffer and 3.0 g of glass beads were used. Tubes were flushed with CO for 30 s and stoppered prior to cell disruption. Crude extracts were obtained by agitating the tubes using a vortex-type mixer at maximum speed for 5 min. Tubes were placed at 4°C and left undisturbed for 10 min to allow CO to equilibrate. Each tube was then centrifuged at 5700 x g for 10 min at 4°C. Supernatants were removed, placed in a 1 cm wide microcuvette, and the absorption spectrum from 390-650 nm was recorded using a Bausch and Lomb Spectronic 2000 spectrophotometer. Multiple extractions were required to remove most of the hemoglobin and performed using the complete extraction procedure as above by resuspending the slurry of glass beads and *Frankia* cells that remained in the tube with 0.5 ml of extraction buffer. In the case of larger hyphal masses (>0.2 g), 1.0 ml of buffer was added. All crude extracts from a given sample were pooled prior to further treatment.

Determination of hemoglobin concentration in culture

The concentration of hemoglobin was determined by modifications of the methods of Tjepkema and Asa (1987). A baseline was drawn on the printout of the absorption spectrum between 405 and 440 nm using a ruler and the difference (ΔA) was measured between this baseline and the HbCO absorption peak at 420/421 nm. To calculate the concentration a ΔE (mM) of 180 was used (Tjepkema and Asa, 1987). It was assumed that the density of the *Frankia* mass was 1.00, and that a negligible amount of culture medium remained in the hyphal mass.

Ion exchange chromatography

Crude extracts from $EAN1_{pec}$ were loaded onto a 10 x 0.9 cm column of DEAE Sepharose CL-6B (Pharmacia Lot No. OE 06415) equilibrated with CO and 0.01 M bis-tris propane (pH 6.9) at room temperature. The entire volume of crude extract was allowed to flow onto the column at a flow rate determined by gravity (approximately 0.5 ml/min). The column was then washed with 10 ml of CO-equilibrated 0.01 M bis-tris propane. Carbonmonoxyhemoglobin was eluted by adding 10 ml CO-equilibrated 0.01M bis-tris propane with 0.5 M NaCl. Ten drop ($\approx$0.4 ml) fractions were collected and those showing an absorption peak near 420 nm were used for pressure cell concentration or molecular weight determination.

Pressure cell concentration

$EAN1_{pec}$ ion exchange fractions containing hemoglobin were pooled and placed in a 10 ml stirred ultrafiltration cell (Amicon model 8010). Gas pressure was applied

using CO to 57.5 psi. A 10.0 kDa pore filter (Millipore Lot # C8E16967) was used for the exclusion membrane. Total volumes between 8-10 ml were concentrated to 1.5-2.0 ml. The sample was then diluted by addition of 8 ml of CO equilibrated KPO_4 buffer. The diluted sample was then concentrated to 1.5 ml to further remove low molecular weight proteins and to reduce the concentration of NaCl remaining from the ion exchange elution buffer. The resulting 1.5-2.0 ml volume was used for determination of optical absorption spectra and the oxygen dissociation rate constant.

Determination of optical absorption spectra

The concentrated sample from the pressure cell containing HbCO was placed in a glass test tube and an enzyme reduction system was added to maintain the heme iron in the ferrous form (Hayashi et al. 1973). The HbCO absorption spectrum between 390-750 nm was immediately recorded using a Beckman DU 7500 Spectrometer. Carbon monoxide was displaced by gently bubbling 100% oxygen through the solution while on ice in the presence of light. The absorption peak near 415/416 nm was then measured. The final HbO_2 spectrum was taken when additional oxygen produced no further changes. Three cycles of this treatment were used to fully convert the ferrous CO-form of the hemoglobin to the oxyferrous form. Because the baseline rose steadily between 500 and 600 nm, α and β wavelength peak values were determined by first obtaining a regression function for the oxy and carboxy spectra between 500 and 600 nm. The regression line for each spectrum was used as the baseline for that spectrum. The residuals determined by regression analysis were plotted against wavelength. These plots

showed the peaks more clearly and were used to assign values to the α and β peak wavelengths for the oxy- and carbonmonoxy spectra.

Oxygen dissociation rate determination

Oxygen dissociation rates (k_{off}) of the $EAN1_{pec}$ hemoglobin from –N and +N cultures were measured using an Applied Photophysics SF17 stopped-flow spectrophotometer as previously described (Cashon et al. 1997). The same $EAN1_{pec}$ hemoglobin samples used to obtain the absorption spectrum were used for the determination of k_{off}. The enzymatic reduction system does not affect the kinetic measurements (Cashon et al. 1997). The hemoglobin from –N and +N cultures was treated in the same way except that for the +N cultures the sample was bubbled with nitrogen to remove any unbound oxygen suspended in the buffer prior to determination of k_{off}. Oxygen was removed from the hemoglobin sample by addition of sodium dithionite solution (2.0 mg/ml). Deoxygenation kinetics were observed by monitoring absorption at 414 nm. Rate constants were determined by standard least squares fitting of the data to a first order equation. The reported value for –N hemoglobin represents the average of six individual trials, while the value for +N hemoglobin represents the average of seven individual trials.

Molecular weight determination

A 30 x 1.1 cm Bio-Gel P-60 (Bio-Rad) column equilibrated with the CO-equilibrated extraction buffer (KPO_4) was used to determine the molecular weight. Ion exchange fractions showing large absorption peaks near 420 nm (hemoglobin fractions)

were used to determine the molecular weight. Hemoglobin fractions of 0.4 ml were combined with 0.25 ml blue dextran 2000 (Pharmacia) solution (10 mg/20 ml CO-equilibrated KPO_4) and passed through the column. Blue dextran 2000 was used to determine the void volume despite cautions against its use as indicated in the Bio-Gel P-Series manual because tests showed that it did not bind to the gel. The molecular weight markers, horse skeletal myoglobin, mol. wt. 17.8 kDa (Sigma) (0.5 mg/ 2 ml CO equilibrated KPO_4 buffer) and cytochrome c, mol. wt. 12.4 kDa; (Sigma) (0.5 mg/ 2 ml CO-equilibrated KPO_4 buffer), were individually combined with 0.25 ml blue dextran 2000 (10 mg/20 ml CO-equilibrated KPO_4 buffer) and passed through the column. The myoglobin sample was run first, followed by the *Frankia* hemoglobin and cytochrome c samples respectively. This sequence was replicated three times. The samples were pumped through the column at a flow rate of 0.1 ml/min and 0.5 ml fractions were collected to determine the elution volume (V_e) and the void volume (V_o). For myoglobin and cytochrome c, the peak of elution was determined by measuring absorbance at 408 nm and 411 nm respectively, while the absorption peak near 420 nm was used for hemoglobin. The internal blue dextran 2000 standard run with each sample was used to determine V_o. The molecular weight of the hemoglobin was determined using a plot of V_e/V_o against the log of the molecular weight (Whitaker, 1963).

Effect of culture age, oxygen, and carbon dioxide

All experimental cultures were inoculated using *Frankia* suspensions grown in the absence of a nitrogen source; the age of inoculum for each experiment is given below. Cultures from one to three flasks containing 125 ml of culture medium were used as the

source of new cultures. When more than one flask was used, the cells were combined prior to homogenization. The cells were homogenized by repeatedly expelling the cells forcefully through the tip of a 10 ml pipette until an even suspension was obtained.

The effect of culture age was examined by growing $EAN1_{pec}$, ArI3, EUN1f, CcI.17, and CcI3 on both nitrogen-free (-N) and nitrogen-supplied (+N) medium. Each strain used 11-20 d old cultures as the inoculum. Each flask was inoculated by adding 3 ml of suspended culture to -N or +N medium in a random order and then randomly assigned to the 14, 28, or 56 d treatment. The *Frankia* strains ArI3, EUN1f, CcI.17, and CcI3 were not examined at 56 d. Four replicates were used for each $EAN1_{pec}$ and ArI3 treatment, while three replicates were used for EUN1f, CcI.17, and CcI3 treatment. The cultures were grown without shaking until harvest.

The effect of CO_2 concentration was examined by growing $EAN1_{pec}$ and CcI3 on –N medium at 0.0, 0.04, 0.2, and 1.0% CO_2. Each $EAN1_{pec}$ flask was inoculated using 2 ml of suspended cells from a 24 d old source. The CcI3 flasks were inoculated using 2 ml of suspended cells from a 30 d old source. The inoculum was added to each flask in a random order and then randomly assigned one of the carbon dioxide treatments. Three replicates were used for each treatment. To obtain 0.0% CO_2, the carbon dioxide was removed from the air pumped in from outdoors by passing it through soda lime. For the 0.04% CO_2 mixture, the air from outdoors was pumped directly to the flasks. The 1.0% CO_2 mixture was made by mixing air from outdoors with compressed CO_2. The 0.2% CO_2 mixture was made by diluting the 1.0% CO_2 with more air from outdoors. Gas sources in each of the above mixtures were individually passed through flowmeters before mixing to control the composition of the respective mixtures. The CO_2

concentrations were checked periodically by gas chromatography using a CARLE analytical gas chromatograph series SX.

The effect of oxygen concentration was examined by growing $EAN1_{pec}$ at 20% and 2% pO_2. Each flask was inoculated using 2 ml of suspended culture from a 50 d old source. The inoculum was added to -N or +N medium in a random order and then randomly assigned one of the oxygen treatments. Three replicates were used for each treatment. The 20% O_2 treatment used atmospheric air pumped in from outdoors. The 2.0% O_2 treatment used the same air combined with compressed N_2. Each gas mixture was supplemented with 0.5% CO_2. The cultures were inoculated and placed on a Burrell Wrist Action Shaker model 75. Both the O_2 and CO_2 concentrations were checked periodically by gas chromatography using a CARLE analytical gas chromatograph series SX.

Data analysis

For the effect of culture age, the hemoglobin concentration and harvested cell mass data collected for each strain were analyzed by two-factor analysis of variance (ANOVA) ($\alpha = 0.05$). For all strains except CcI3, the assumptions of the model, normality of error terms and constant variance, were met without transforming the data. In the case of CcI3, the data were log transformed.

For the effect of carbon dioxide, the harvested cell mass data were analyzed by single factor ANOVA ($\alpha = 0.05$). For the effect of oxygen, analysis of variance was not performed because the time of cell growth differed between the -N and +N experiments.

The means separations for the effect of culture age and effect of carbon dioxide were determined by Tukey's multiple pairwise comparisons ($\alpha = 0.05$). For the effect of pO_2 experiment, differences between means within each nitrogen treatment were determined by t-tests ($\alpha = 0.05$). All statistics were performed using SYSTAT for Windows version 9.0.

Results

Presence of hemoglobin and vesicles

All of the five *Frankia* strains used in this study (shown in Table 1) were found to produce hemoglobin (concentrations discussed below). These strains comprise a diverse group representing all three of the genetically identified host specificity groups (*Elaeagnaceae*, *Alnus*, and *Casuarinaceae*) with each strain belonging to a different genomic group (An et al. 1985; Fernandez et al. 1989; Akimov and Dobritsa, 1992; Lumini and Bosco, 1999).

Vesicles were observed for all five *Frankia* strains whether grown on –N or +N medium. At 14 d and 28 d, vesicles were more numerous in –N cultures than in +N. At 28 d, all strains had abundant, well-formed vesicles when grown in absence of nitrogen. Vesicles were absent only in CcI3 +N cultures at 28 d. Vesicles have been observed for $EAN1_{pec}$ and EUN1f when grown on medium containing double the concentration of nitrogen used in this study (Tisa and Ensign, 1987a). For $EAN1_{pec}$ observed at 56 d, vesicles were seen for both treatments, the –N cultures having more.

Carbonmonoxy and oxyhemoglobin absorption spectrum

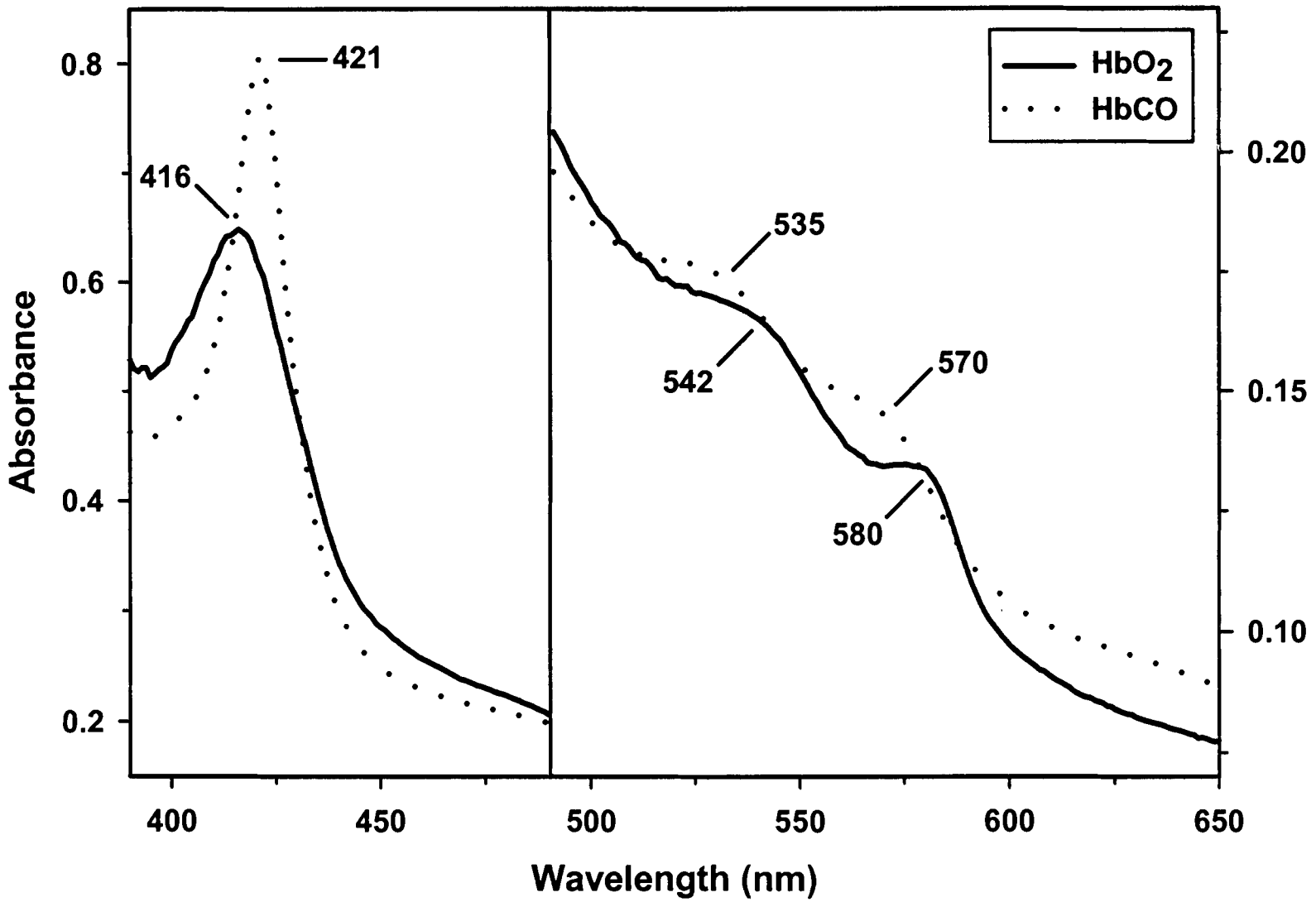
Figures 1 and 2 show the absorption spectra for the hemoglobin purified from *Frankia* strain $EAN1_{pec}$ grown on –N and +N media. The spectra of the carbonmonoxyhemoglobin showed three distinct absorption peaks: 420/421 nm, 535/542 nm, and 570/569 nm for –N and +N samples, respectively. Removal of carbon monoxide and addition of oxygen to form oxyhemoglobin showed a shift in the maxima of these peaks to 416 nm, 542/545 nm (–N and +N samples, respectively), and 580 nm. Addition of carbon monoxide to oxygenated samples returned the peaks to the values originally observed.

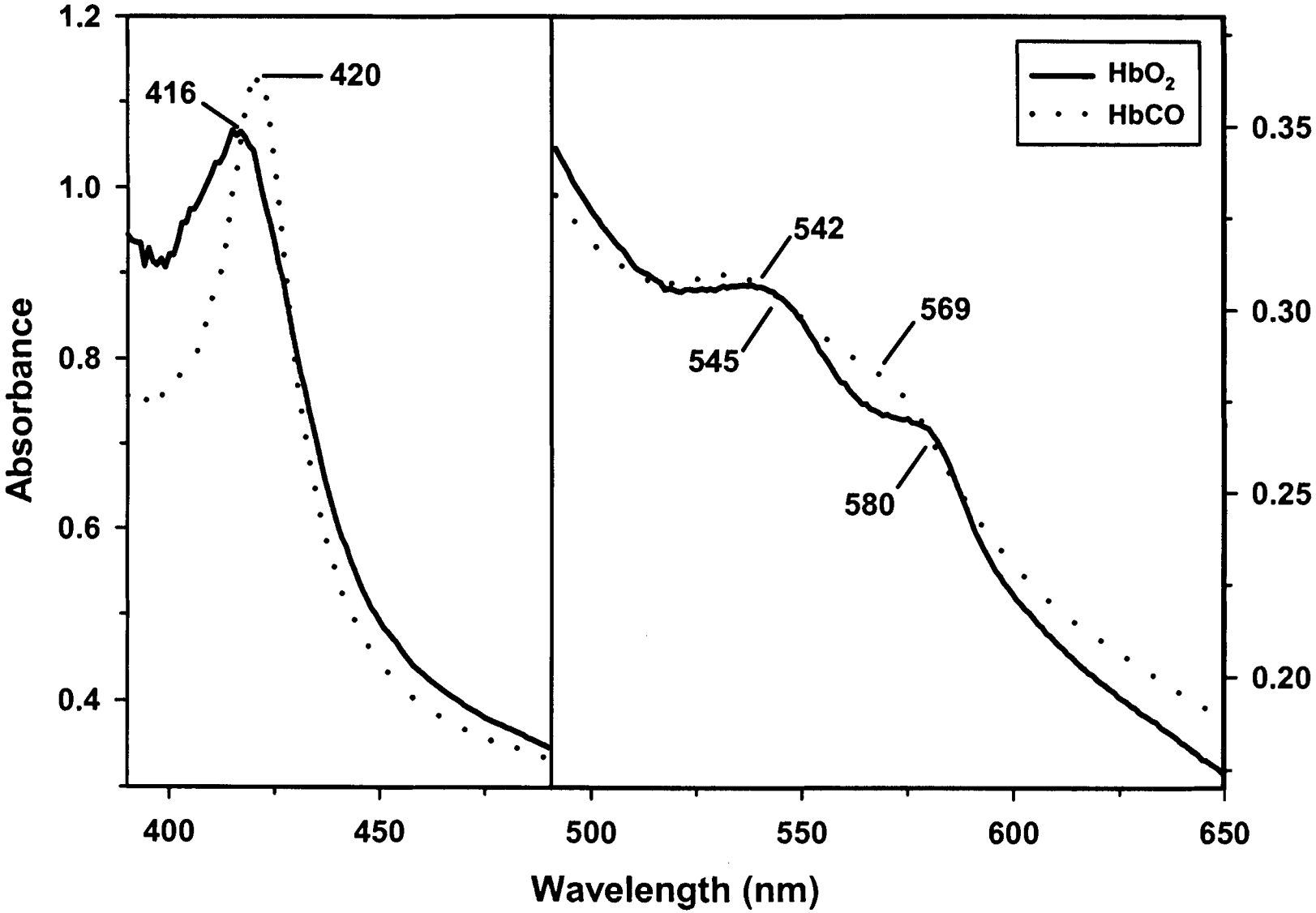
Older cultures of $EAN1_{pec}$ and the crude extracts were dark red to crimson in color. The crude extracts from +N cultures had a darker color than the –N culture crude extracts. The absorption spectra of the crude extracts were measured between 390 and 650 nm. A broad peak at 474 nm was observed, which approached the amplitude of the carbonmonoxy peak at 420/421 nm. The 474 nm peak was almost entirely removed from the hemoglobin samples using ion exchange.

Molecular weight and oxygen dissociation rate constant

The molecular mass of the –N $EAN1_{pec}$ samples as determined by size exclusion chromatography was 13.4 ± 0.2 kDa (± SE, n = 3).

This hemoglobin formed a stable complex in the carbonmonoxy form. At room temperature, the –N carbonmonoxyhemoglobin showed no measurable loss over the course of 1 h as viewed by the absorption spectrum.


HbO2
HbCO
421
416
535
542
570
580
Absorbance
0.8
0.6
0.4
0.2
0.20
0.15
0.10
400
450
500
550
600
650
Wavelength (nm)


1.2
1.0
0.8
0.6
0.4
Absorbance
416
420
542
545
569
580
HbO2
HbCO
0.35
0.30
0.25
0.20
400
450
500
550
600
650
Wavelength (nm)

When samples were gently bubbled with air or 100% O_2 at room temperature or 4°C, however, degradation was observed in a matter of minutes (data not shown). This was remedied by addition of the enzyme reducing system (Hayashi et al. 1973) and allowed measurements of the oxygen dissociation rate constant (k_{off}). The k_{off} for hemoglobin was 131.2 s^{-1} ± 5.805 (± SE, n = 6) for the –N culture sample and 166 s^{-1} ± 8.188 (± SE, n = 7) for the +N culture sample. These are significantly different from one another ($p = 0.0064$).

Cell growth

Frankia have very slow doubling times (15 h or more) (Benson and Silvester, 1993). The present data for $EAN1_{pec}$ show a linear, non-exponential growth rate over the 14-56 d period (Figure 3). This implies that the cells are no longer in their exponential growth phase when collected at 14 d and some senescent cells are probably present in the culture when harvested. In static cultures, cells clumped and formed small spheres. These spheres became larger and more numerous with age.

Effect of culture age on hemoglobin concentration in five strains of *Frankia*

When the effect of age and nitrogen were examined, three-factor analysis of variance indicated that all factor levels (strain, nitrogen status, and age of culture) were significant ($p < 0.001$) in predicting both hemoglobin concentration and harvested cell mass. Because the effect of strain was significant, data from individual strains were analyzed by two-factor analysis of variance. Mean comparisons were performed and are shown in Tables 2 and 3.

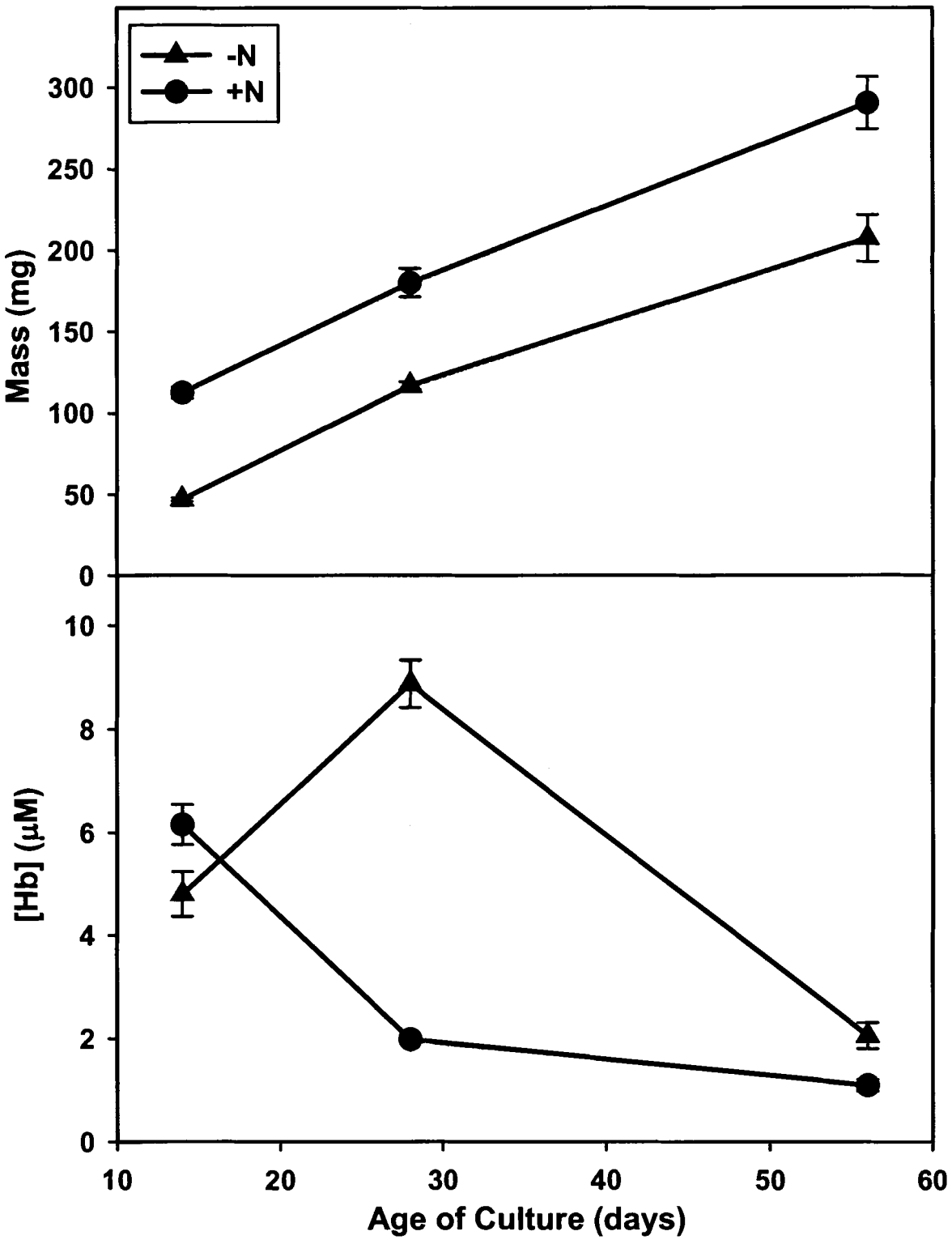

-N
+N
Mass (mg)
300
250
200
150
100
50
0
[Hb] (μM)
10
8
6
4
2
0
10
20
30
40
50
60
Age of Culture (days)

Table 2. Effect of *Frankia* strain, time of harvest, and availability of nitrogen in the growth medium on hemoglobin concentration (μM) in cultured *Frankia*.

Media	-N		+N	
Harvest date	14 d	28 d	14 d	28 d
Strain				
$EAN1_{pec}$	4.81 ± 0.44 B	8.88 ± 0.45 C	6.16 ± 0.39 B	2.00 ± 0.01 A
ArI3	5.13 ± 0.05 A	6.18 ± 0.19 B	10.07 ± 0.15 D	7.60 ± 0.09 C
EUN1f	5.36 ± 0.19 A	7.54 ± 0.72 AB	9.53 ± 0.27 B	7.78 ± 0.83 AB
CcI.17	4.94 ± 0.21 B	9.08 ± 0.82 C	4.02 ± 0.36 AB	2.35 ± 0.16 A
CcI3[†]	2.38 ± 0.36 A	22.78 ± 2.16 C	7.82 ± 0.42 B	8.22 ± 0.38 B

Note: Cultures were grown for 14 d or 28 d on nitrogen-free (-N) or nitrogen-supplied (+N) medium. Values are means ± SE (n=4 for $EAN1_{pec}$ and ARI3; n=3 for EUN1f, CcI.17, and CcI3). Within rows, means followed by different letters are significantly different ($p \leq 0.05$). [†] CcI3 data was log transformed to fit the ANOVA model assumptions; actual means are listed in the table.

Table 3. Effect of *Frankia* strain, time of harvest, and availability of nitrogen in the growth medium on freshly harvested cell mass (mg) in cultured *Frankia*.

Media	-N		+N	
Harvest date	14 d	28 d	14 d	28 d
Strain				
$EAN1_{pec}$	47.25 ± 0.99 A	116.95 ± 2.37 B	112.68 ± 3.49 B	180.20 ± 8.79 C
ArI3	37.60 ± 2.63 A	84.93 ± 4.37 C	44.20 ± 3.25 A	63.80 ± 3.24 B
EUN1f	23.80 ± 1.04 A	36.30 ± 1.52 B	31.20 ± 3.09 AB	48.37 ± 2.00 C
CcI.17	34.63 ± 1.87 B	59.10 ± 4.26 C	26.70 ± 1.74 AB	18.17 ± 4.48 A
CcI3[†]	42.43 ± 3.52 A	57.27 ± 3.14 AB	71.20 ± 4.10 B	115.83 ± 16.11 C

Note: Cultures were grown for 14 d or 28 d on nitrogen-free (-N) or nitrogen-supplied (+N) medium. Values are means ± SE (n=4 for $EAN1_{pec}$ and ARI3; n=3 for EUN1f, CcI.17, and CcI3). Within rows, means followed by different letters are significantly different ($p \leq 0.05$). [†] CcI3 data was log transformed to fit the ANOVA model assumptions; actual means are listed in the table.

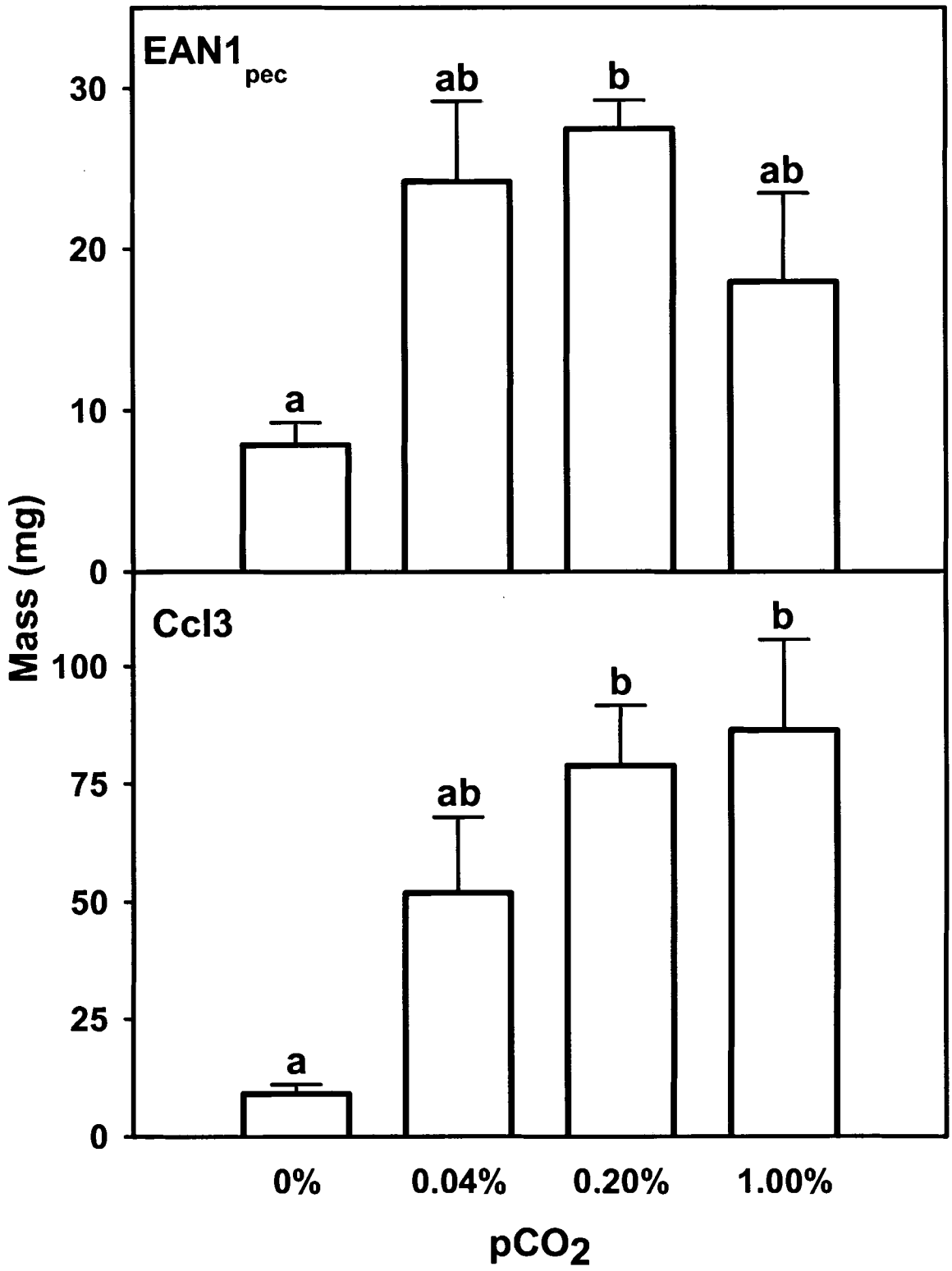
For –N treatments, the hemoglobin concentration in strains $EAN1_{pec}$, ArI3, CcI.17, and CcI3 significantly increased from 14-28 d. Only EUN1f did not show a significant increase in hemoglobin concentration from 14-28 d on –N medium. The greatest increase in hemoglobin concentration from 14-28 d occurred in the CcI3 cultures, which showed a nearly 10-fold increase. For the $EAN1_{pec}$ strain, hemoglobin concentration peaked at 28 d, but returned to low levels at 56 d (Figure 3).

For +N treatments, two strains showed a significant decrease in hemoglobin concentration from 14-28 d, while three strains showed no statistically significant change over the same time period. For the $EAN1_{pec}$ strain, a decline hemoglobin concentration is observed from 14 to 56 d (Figure 3).

Cell mass increased in nearly all cultures between 14 and 28 d. While the –N cultures of CcI3 and the +N cultures of CcI.17 (Table 3) did not increase in this time period, these showed no significant change.

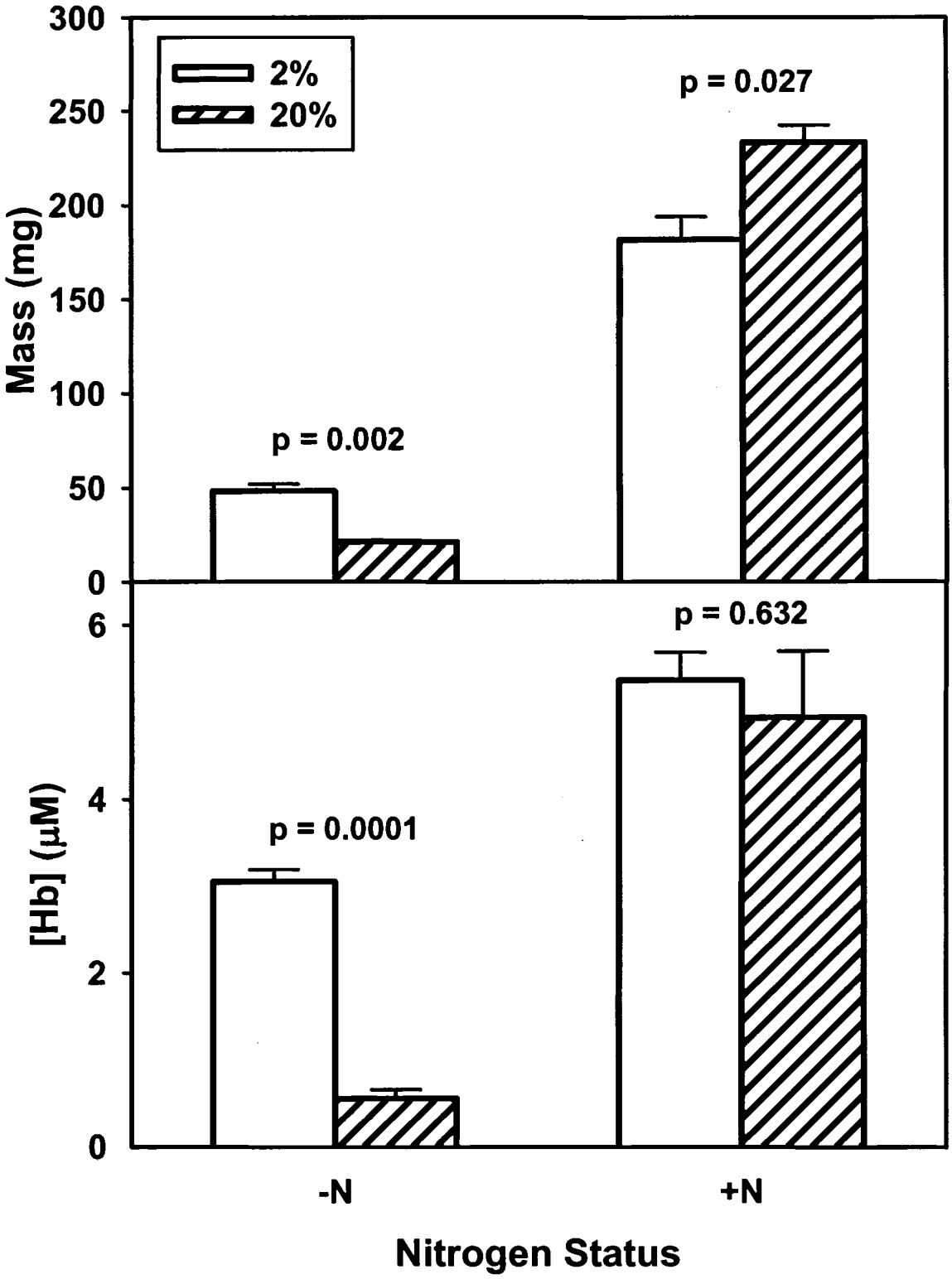
Effect of carbon dioxide

The fresh cell masses of $EAN1_{pec}$ and CcI3 grown at 0.0%, 0.04%, 0.2%, and 1.0% CO_2 are shown in Figure 4. The cell mass of the 0.0% CO_2 treatment was significantly lower than the 0.2% CO_2 treatment in both strains. This experiment was repeated for $EAN1_{pec}$ and yielded similar results (data not shown). Visual observations of the cultures suggested that the effects of CO_2 were more pronounced during the first few days of culture, when internal generation of CO_2 by respiration was lowest.


EAN1pec
30
20
10
0
a
ab
b
ab
Mass (mg)
Ccl3
100
75
50
25
0
a
ab
b
b
0%
0.04%
0.20%
1.00%
pCO2

Effect of oxygen

The hemoglobin concentration and harvested cell mass of -N and +N cultures grown at 2% or 20% O_2 are shown in Figure 5. For the –N cultures, the mass was more than two times greater at 2% O_2 than at 20% O_2, while the hemoglobin concentration was nearly six times greater at 2% O_2 than at 20% O_2. The +N cultures showed somewhat more growth at 20% O_2 than at 2% O_2, but no significant difference in hemoglobin concentration. A second experiment was performed as above except each of the treatments were harvested at an earlier age (8 d for –N, 5 d for +N). This produced similar results (data not shown).


300
250
200
150
100
50
0
Mass (mg)
2%
20%
p = 0.027
p = 0.002
6
4
2
0
[Hb] (μM)
p = 0.632
p = 0.0001
-N
+N
Nitrogen Status

Discussion

Experimental considerations

All cultures were inoculated with two or three ml of suspended –N cells. Thus the question arises whether the inoculum is the source of some of the vesicles and hemoglobin observed in the +N cultures. Since the cells used as inoculum represent less than 10% of the total harvested cell mass after 14 d and 28 d of growth, they had little effect on the results.

Vesicles and cell growth

The harvested cell mass shows that cell growth was generally greater on +N medium for each age of culture treatment (Table 3). This is consistent with cellular energy being used in nitrogen fixation and to form secondary structures, such as vesicles, in the nitrogen-fixing cultures, rather than the energy being used for cell proliferation.

The fact that vesicles are observed at low frequency in the presence of a nitrogen source may indicate that a small degree of nitrogen fixation is occurring during the early exponential growth of the +N cultures. It has been suggested that vesicles present in some strains supplied with nitrogen are an agent of quicker response time when nitrogen fixation is necessary (Tisa and Ensign, 1987a). Alternatively, ammonia-supplied cultures of CcI.17 showed no nitrogenase activity, despite the presence of vesicles (Meesters et al. 1985).

Absorption spectrum

The α, β, and Soret peak wavelengths are very similar for the hemoglobin samples from both –N and +N cultures and are consistent with the spectra observed for

the truncated hemoglobin found in *Nostoc commune* (Thorsteinsson et al. 1999), *Paramecium* (Das et al. 2000), and *Mycobacterium tuberculosis* (Couture, Yeh, et al. 1999). The hemoglobin reported in *Chlamydomonas eugametos* (Couture and Guertin, 1996), *Synechocystis* PCC 6803 (Couture et al. 2000), and *Frankia* strain CcI3 (Tjepkema et al. 2002) also show similar oxyhemoglobin spectra. These last three show a combined α/β peak in the carbonmonoxy form with absorbance values similar to the two separate α and β peaks in $EAN1_{pec}$ hemoglobin. In the oxyhemoglobin spectra of all the GLBN truncated hemoglobins, the intensity of the α peak is always less than that of the β peak (Couture and Guertin, 1996; Couture, Yeh, et al. 1999; Couture et al. 2000; Tjepkema et al. 2002). However, the pronounced upward slope of the absorbance baseline from 450 to 650 nm (Figures 1 and 2) is not typical of hemoglobins, and may be due to impurities remaining in the $EAN1_{pec}$ hemoglobin sample, as discussed below.

Pigment in $EAN1_{pec}$ hemoglobin samples

The more concentrated hemoglobin crude extracts had a dark red color. This is probably due to 2-methyl-4,7,9,12-tetrahydroxy-5,6-dihydrobenzo[a]naphthacene-8,13-dione, a pigment previously found in static, month-old cultures of $EAN1_{pec}$ (Gerber and Lechevalier, 1984). This is a natural quinone with an absorption peak at 470 nm (Gerber and Lechevalier, 1984); this is consistent with our finding a peak at 474 nm. Although similar quinones are carcinogenic, this one is not (Gerber and Lechevalier, 1984). Quinones can react with terminal amino groups and sulphydryl groups of proteins. While the pigment molecule should pass through the 10 kDa membrane used in pressure cell concentrations, the pigment attached to a protein may be present in these samples. This

could be the cause of the upward sloping absorption spectrum between 450-650 nm observed in Figures 1 and 2. This pigment has been reported to have pH and redox indicator properties and may be located on a 17 kilobase plasmid found in $EAN1_{pec}$ as observed by loss of the pigment when grown on novobiocin, a plasmid-curing agent (Tisa et al. 1999).

Oxygen kinetics of *Frankia* hemoglobin

The oxygen dissociation rate constants from both $EAN1_{pec}$ samples are faster than any of the reported GLBNs (Table 4). This is approximately three-fold faster than that of *Frankia* strain CcI3 (Tjepkema et al. 2002), and approximately two-fold faster than *Nostoc* (Thorsteinsson et al. 1999). If the hemoglobin from *Frankia* is indeed a member of the GLBN group, this would create an interesting sub-group; the truncated hemoglobins from the nitrogen-fixing members would have the fastest oxygen dissociation rates. Furthermore, *Nostoc* hemoglobin has a histidine residue at position B10 of its tertiary structure (Couture, Yeh, et al. 1999) rather than the tyrosine found in this position in the more than 40 other truncated hemoglobins from 32 different organisms (Wittenberg et al. 2001). The near ubiquitous tyrosine residue at B10 in the truncated hemoglobins is able to form a strong hydrogen bond with the E7-Gln, thus providing extra stability to the hydrogen bond network (Das et al. 2001). The suspicious absence of the B10-Tyr in *Nostoc* may contribute to the faster dissociation rate. A distinct sub-group would be supported if the hemoglobin from *Frankia* also lacks the B10-Tyr.

Table 4. Characteristics of *Frankia* hemoglobin and the truncated hemoglobins from the GLBN group.

Organism	Mass (kDa)	k'_{on} ($\mu M^{-1} s^{-1}$)	k_{off} (s^{-1})	$K_d = k_{off}/k'_{on}$ (nM)	P_{50} (mmHg)	References
Frankia strain $EAN1_{pec}$ (-N)	13.4		131			This study
Frankia strain $EAN1_{pec}$ (+N)			166			This study
Frankia strain CcI3	14.1	206	56	274		Tjepkema et al. 2002
Nostoc commune	12.5	390	79	203	0.55	Potts et al. 1992; Thorsteinsson et al. 1999
Paramecium sp.	≈13	30	25	838	0.45-0.6	Smith et al. 1962; Iwaasa et al. 1989; Das et al. 2000
Tetrahymena sp.	≈14.3				0.2	Iwaasa et al. 1990; Takagi et al. 1993; Korenaga et al. 2000
Chlamydomonas eugametos	16[†]	$> 10^7$	0.014		0.005	Couture et al. 1994; Couture, Das, et al. 1999
Mycobacterium tuberculosis	14.4	25	0.2		0.013	Couture, Yeh, et al. 1999
Synechocystis PCC 6803	13.7		0.011			Kaneko and Tabata, 1997; Couture et al. 2000; Scott and Lecomte, 2000

[†] The mature hemoglobin (16 kDa) includes 38 amino acids which act as a transit peptide for chloroplast import (Couture et al. 1994).

The association rate of the cyanobacterial hemoglobin is the highest reported in a study of non-vertebrate hemoglobins (Thorsteinsson et al. 1999; Weber and Vinogradov, 2001). This, coupled with a relatively fast dissociation rate, gives the cyanobacterial hemoglobin a moderate oxygen affinity. The same observation is made when comparing *Frankia* strain CcI3 hemoglobin. These rates are consistent with hemoglobin functioning in facilitation of oxygen transport over short distances, as in the vesicle of *Frankia* or heterocyst of *Nostoc* (Tjepkema et al. 2002). In this manner, respiration could proceed even at the low oxygen tensions required for nitrogenase activity. If this is indeed the function of hemoglobin in $EAN1_{pec}$, the oxygen association rate constant may exceed or be near to the value observed for *Nostoc* hemoglobin and calculated for *Frankia* strain CcI3 hemoglobin.

Culture age and hemoglobin concentration

The calculation of hemoglobin concentration includes the entire harvested cell mass. If some of the cells become senescent over time, and hemoglobin is lost from those cells, those biologically inert cells still contribute to the harvested cell mass. Thus a decrease in hemoglobin concentration over time could be observed even if the level of hemoglobin expression remained the same in the active cells making hemoglobin. An increase in hemoglobin concentration over time may indicate up-regulation of hemoglobin expression even greater than indicated by the proportional gain, since only the biologically active cells would be expressing the protein.

In –N cultures, there was an increase in hemoglobin concentration from 14 d to 28 d in four of the five strains (Table 2). In contrast, the hemoglobin concentration in the

+N cultures decreased or did not change during this time period. The cause of this difference is not clear. The dramatic decrease in hemoglobin concentration from 28-56 d in the nitrogen-fixing treatments of $EAN1_{pec}$ (Figure 3) may have been due to senescence by many of the cells with only a small amount of the biomass being active.

Effect of carbon dioxide

The carbon dioxide experiment (Figure 4) showed an increased rate of growth at 0.2% CO_2 when compared to 0.0% CO_2. As a result, carbon dioxide was added to all the gas mixtures in the oxygen experiment (Figure 5) as described in the Materials and Methods. The beneficial effects of carbon dioxide on growth suggest that addition of carbon dioxide in the culturing of *Frankia* at low initial densities when little carbon dioxide is being generated from cellular respiration would enhance growth. In particular, carbon dioxide addition might aid in the success of *Frankia* isolation from root nodules.

Effect of oxygen concentration on hemoglobin concentration

In *Nostoc*, hemoglobin was found only under conditions of reduced oxygen supply (Potts et al. 1992; Hill et al. 1996). This is not the case for the +N cultures of *Frankia*, where there was no difference in hemoglobin concentration between cultures grown at 2% and 20% oxygen (Figure 5). In contrast, there was a much higher hemoglobin concentration in –N cultures at 2% than at 20% oxygen. Thus, a reduced oxygen concentration does increase hemoglobin in –N cultures. The absolute concentration of hemoglobin in the –N cultures at 20% oxygen, however, was very low and the cell mass was also very low. It thus seems possible that the low hemoglobin

concentration in these –N, 20% oxygen cultures was related to a very low growth rate. The +N cultures had a much higher hemoglobin concentration and cell mass. This result supports the possibility that hemoglobin concentration is highest in the most rapidly growing cells, and that the effects of ammonium addition and oxygen are indirect, acting via their effects on growth rate.

Association between hemoglobin concentration and nitrogen fixation

The gene encoding the *Nostoc* hemoglobin is located within the *nif* operon, which contains the genes responsible for nitrogen fixation (Potts et al. 1992). Furthermore, a specific transcription factor (NtcA) essential for heterocyst formation binds immediately upstream of *glbN* in *N. commune* UTEX 584 (Hill et al. 1996). The hemoglobin gene location with respect to the *nif* operon within the *Frankia* genome is unknown. While the nitrogen fixing gene cluster in *Frankia* has multiple open-reading frames, a hemoglobin was not reported (Harriott et al. 1995; McEwan and Gatherer, 1998).

In contrast to *Nostoc*, the present results with *Frankia* do not strongly support an association between nitrogen fixation and hemoglobin concentration. The differences in hemoglobin concentration between -N and +N cultures shown in Table 2 are not large. At 14 d, the hemoglobin concentration in +N cultures is actually higher than in the –N cultures in three of the five strains. At 28 days, the hemoglobin concentration is greater in the –N cultures in three of the five strains, but this could be due to senescent cells in the +N cultures. The best support for elevated hemoglobin concentrations in nitrogen-fixing cells comes from the data for strain CcI3, where the concentration in –N cultures at 28 d is almost three times higher than in the +N cultures at either 14 or 28 d.

Possible function of *Frankia* hemoglobin

The occurrence of moderately high concentrations of hemoglobin in the +N cultures of *Frankia*, with no difference between +N cultures bubbled with 2% or 20% oxygen (Table 2 and Figure 5) suggests a function other than facilitation of oxygen diffusion and one that is not specific to nitrogen fixation. The other possibility is that hemoglobin is constitutively expressed in *Frankia*, and is thus present under conditions of high oxygen and fixed nitrogen, even though it has no function under these conditions. This situation could possibly exist due to the symbiotic nature of *Frankia*, that of nitrogen fixation within the oxygen-limited root nodule, where fixed nitrogen is not available and constitutive expression of hemoglobin under nitrogen-fixing conditions might be of little disadvantage.

The function of hemoglobin in the +N cultures bubbled with 20% oxygen is not clear. Hemoglobin might possibly function as a terminal oxidase in respiration, but its advantage over typical terminal oxidases is unclear under conditions of ample oxygen supply.

Under oxygen-limiting conditions, *Nostoc* hemoglobin has been proposed to gather oxygen within the cell and present it to the terminal oxidase (Hill et al. 1996; Thorsteinsson et al. 1999). This could enhance respiration both in support of nitrogen fixation and overall metabolism. The observation of hemoglobin in the absence of vesicles in CcI3 in the +N, 28 d treatment suggests that hemoglobin is not localized solely within vesicles for this strain. In fact, hemoglobin in *Nostoc* is found both in vegetative cells and heterocysts (Hill et al. 1996). Moreover, it is concentrated in the periphery of the cells, where it could interact with respiratory enzymes.

Such gathering of oxygen would be a form of facilitated diffusion of oxygen. For hemoglobin to facilitate significant oxygen diffusion through a cell, there must be a gradient of oxygenated hemoglobin between the oxygen source and the point of oxygen consumption. Further, the concentration must be high enough that oxygen transport via hemoglobin is substantial and more efficient than that of free oxygen diffusion (Hill, 1998). Wittenberg (1992) proposed a concentration of no less than 100 μM would be necessary to perform this function. None of the *Frankia* strains approach this concentration. If the hemoglobin were mostly confined to a small percentage of the total cell mass, such as the nitrogen-fixing vesicle, the concentration of hemoglobin may be sufficient to function in facilitated diffusion. This role for *Frankia* strain CcI3 hemoglobin was previously proposed by Tjepkema et al. (2002). Localization at the periphery of vegetative cells in *Nostoc* would also increase the concentration. The very rapid oxygen dissociation and association rates of *Frankia* may also reduce the concentration of hemoglobin needed to deliver oxygen to the cytochrome oxidase.

Conclusions

In contrast to the cyanobacteria, hemoglobin has been found in all strains of *Frankia* studied to date (CcI3, $EAN1_{pec}$, ArI3, CcI.17, and EUN1f). Since these strains are from genetically distinct taxonomic groups (see Table 1), it is quite possible that hemoglobin is found in many or all *Frankia* strains. Based on the similarity in molecular mass and oxygen dissociation of the hemoglobin from *Frankia* strains CcI3 and $EAN1_{pec}$ to *N. commune* hemoglobin, it is likely that *Frankia* hemoglobin is another member of the GLBN group of truncated hemoglobins. Although the oxygen association rate and

oxygen affinity of $EAN1_{pec}$ hemoglobin are unknown, it seems likely that its function is similar to the hemoglobins from *N. commune* and *Frankia* strain CcI3. The high rate of oxygen dissociation is consistent with a role in oxygen transport over short distances.

For all strains tested, the concentrations of hemoglobin measured are not sufficient to perform a function in facilitated diffusion if the hemoglobin is evenly distributed. If the hemoglobin were concentrated in certain parts of the cells, i.e. the vesicles, then the hemoglobin concentration in the area where nitrogen fixation occurs could be great enough to perform a function in facilitated diffusion.

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